American Journal of Medical Genetics 65:320–324 (1996)

Unusual Thermolability Properties of Beta-Hexosaminidase: Studies of Enzyme From Cultured Cells and Clinical Implications

Elizabeth M. Prence, Irena Zalewski, and Marvin R. Natowicz

Division of Medical Genetics, E.K. Shriver Center, Waltham (E.M.P., I.Z., M.R.N.), Departments of Neurology (E.M.P., M.R.N.), and Pathology (M.R.N.), Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

Tay-Sachs disease (TSD) is a neurodegenerative genetic disorder caused by a deficiency of beta-hexosaminidase A (Hex A) activity. To diagnose TSD and to screen for TSD heterozygosity, laboratories use an assay that exploits the differential thermolability of the major beta-hexosaminidase isoenzymes, Hex A and Hex B. At 50–52°C Hex A is labile, and Hex B is stable. We previously noted that the stability of leukocyte Hex B at 52°C varied significantly, depending on the sample concentration in the incubation mixture. We have now examined this phenomenon in enzyme from cultured cells used for prenatal and postnatal diagnostic testing. We found that fibroblast Hex A and Hex B behave similarly to the leukocyte isoenzymes. In control and TSD fibroblasts there was a linear correlation between Hex B thermostability and sample concentration; at lower sample concentrations Hex B was less stable than at higher concentrations. Dialysis of the samples prior to heat treatment did not change the thermostability properties of Hex B, indicating that the change in stability is not due to a soluble low molecular weight substance. Cultured amniotic fluid cell and chorionic villus cell Hex B had a similar, but less pronounced, instability at low sample concentrations. Therefore, the unusual thermolability properties of Hex B, first detected for leukocyte Hex B, were noted in multiple tissues. Based on these data, we suggest that the concentration of cell extract be stringently controlled when the heat-inactivation method is used for the pre- or postnatal diagnosis of TSD, and that supplementation with non-thermolability-based beta-hexosaminidase assays should be employed as needed.

KEY WORDS: beta-hexosaminidase, Tay-Sachs disease, prenatal diagnosis

INTRODUCTION

Tay-Sachs disease (TSD) is an autosomal recessive neurodegenerative disorder caused by a deficiency of the lysosomal enzyme beta-hexosaminidase A (E.C. 3.2.1.52) that, in turn, results in neuronal storage of G_{M2} ganglioside [reviewed in Gravel et al., 1995]. TSD occurs with increased frequency among individuals of several ethnic groups or geographic regions, such as individuals of Ashkenazi Jewish ethnicity. Consequently, heterozygote screening and prenatal diagnosis are routinely offered to high-risk individuals and couples [Kaback et al., 1993].

Testing for heterozygosity or homozygosity for TSD consists of measurement of the two major isoenzymes of human beta-hexosaminidase, beta-hexosaminidases A and B (Hex A and Hex B), both of which occur in blood and other tissues. Hex A is a dimer of an alpha and a beta subunit of Hex, whereas Hex B is a homodimer of beta subunits [Gravel et al., 1995]. The A isoenzyme is capable of hydrolyzing terminal, nonreducing, beta-linked N-acetylgalactosamine from negatively charged substrates (e.g., G_{M2} ganglioside) as well as from uncharged substrates (e.g., oligosaccharides); the B isoenzyme hydrolyzes uncharged substrates. The alpha and beta subunits are coded for by different genes; TSD is caused by mutations in the alpha subunit gene that result in deficiency of Hex A. Mutations in the beta subunit gene are responsible for a related disorder, Sandhoff disease, which is characterized by a deficiency of both Hex A and Hex B activities [Gravel et al., 1995].

The most common clinical method for Hex analysis is based on the differential thermolabilities of Hex A and Hex B [Kaback et al., 1977; Shapira et al., 1989]. An

Received for publication October 10, 1995; revision received April 9, 1996.

Address reprint requests to Dr. Elizabeth Prence, Division of Medical Genetics, E.K. Shriver Center, 200 Trapelo Road, Waltham, MA 02254.

artificial fluorescent substrate, 4-methylumbelliferyl-2-deoxy-2-acetamido-beta-D-glucopyranoside, is used most often for these determinations. This substrate can be hydrolyzed by both Hex A and Hex B. However, because Hex A activity is more sensitive to heat, incubation of a sample at 50–52°C inactivates the Hex A, leaving only Hex B activity. Therefore, performing the enzyme assay on sample aliquots with and without prior heat treatment provides activity measurements for the total hexosaminidase (A plus B) and for Hex B alone. The diagnosis of TSD and heterozygosity for TSD is based on the percentage of total hexosaminidase that is due to Hex A (calculated as 100% − % of Hex B) [Kaback et al., 1977; Shapira et al., 1989].

In a previous study, we documented an unusual behavior of leukocyte Hex in a heat-inactivation assay [Prence et al., 1993]. Specifically, significantly different results were obtained for the percentages of Hex A and Hex B depending on the amount of leukocyte sample used in the assay. There was an inverse relationship between the amount of sample and the percentage of Hex A observed; the higher the sample concentration, the lower the percentage of Hex A. We determined that this apparent change in Hex A was due to alterations in Hex B activity. Since the Hex A value is calculated from the measured Hex B activity, any change in Hex B would appear as an opposite change in Hex A. Our data indicated that at low sample concentrations Hex B was unstable and became partially inactivated during heat treatment. This is, to the best of our knowledge, an unusual enzymatic property unique to beta-hexosaminidase.

An important issue that needed to be addressed concerned whether this is a tissue-specific property of leukocyte Hex B or if it occurs in other cell types as well. We have now extended our earlier study and evaluated beta-hexosaminidase thermolability properties in cultured fibroblasts, amniotic fluid cells, and chorionic villus cells. In this report we show that the unusual thermostability of leukocyte Hex B also occurs with Hex B of cultured cells. The significance of these results is discussed.

MATERIALS AND METHODS

Reagents

4-methylumbelliferyl-2-deoxy-2-acetamido-beta-D-glucopyranoside (MUG) and 4-methylumbelliferone were from Sigma Chemical Co. (St. Louis, MO). 4-methylumbelliferyl-N-acetylglucosamine-6-sulfate (MUGS) was purchased from the HSC Research and Development Ltd. Partnership (Toronto, Canada). Dialysis tubing (12,000–14,000 molecular weight cutoff) was from Spectrum Medical Industries, Inc. (Houston, TX).

Samples

Cultured fibroblasts were obtained from the Human Mutant Cell Repository (Camden, NJ) and from patients diagnosed with Tay-Sachs or Sandhoff disease in our laboratory. Amniotic fluid cells (AFCs) were sent to our laboratory for prenatal diagnosis of TSD. Chorionic villus sample (CVS) cells were obtained from Dr. E. Grebner of the Philadelphia Tay-Sachs Disease Prevention Program, Division of Medical Genetics, Thomas Jefferson Medical Center, Philadelphia, PA.

Beta-Hexosaminidase Assays

Heat-stable and total Hex activities in cultured cells were determined by the heat-inactivation method, using MUG as substrate [Kaback et al., 1977; Shapira et al., 1989], as described below. Cell pellets were sonicated in cold, distilled water and then centrifuged at 200*g* for 10 min. The supernatant was removed and used for the assay. Aliquots were diluted with bovine serum albumin (2mg/ml) in citrate-phosphate buffer, and then incubated at 50°C for 2 hr prior to assay. Beta-hexosaminidase activity was determined fluorometrically in the untreated and heat-treated samples by incubation with MUG in citrate-phosphate buffer, pH 4.4, for 6 min at 37°C. Total hexosaminidase refers to the activity measured in the unheated sample. Hex B is the activity measured in the heat-treated sample. The activity of Hex B divided by the total activity (× 100%) gives the percent Hex B value. Percent Hex A is equal to 100 minus the percent Hex B.

Hex A was also assayed directly with MUGS, a Hex A-specific substrate, as previously described [Prence et al., 1993]. Protein concentration of cell sonicates was determined according to the method of Lowry et al. [1951].

RESULTS

We extended our previous study of the unusual thermal stability behavior of beta-hexosaminidase to include evaluations of the enzyme from cultured cells. Normal control, TSD, and Sandhoff disease fibroblasts were assayed for Hex activity at varying sample concentrations (5–20 μg protein per assay). As shown in Figure 1A, when normal control fibroblasts were tested, we observed a difference of approximately 8 percentage points of Hex A activity between the lowest and highest sample concentrations tested, similar to what was observed in leukocytes [Prence et al., 1993]. TSD fibroblast Hex behaved similarly (Fig. 1B), as the percent Hex A varied greatly depending on the amount of sample in the assay.

In contrast, when Sandhoff disease fibroblasts were analyzed there was no difference in the percent Hex A activity obtained at the various sample concentrations (Fig. 1C), again similar to what we observed in leukocytes [Prence et al., 1993]. Sandhoff disease is clinically similar to Tay-Sachs disease, but is due to a mutation in the gene for the beta subunit of Hex [Gravel et al., 1995]. A typical Sandhoff disease heterozygote has a lower-than-normal total amount of Hex activity, but the percent Hex A is much higher than normal [Kolodny, 1972; Lowden et al., 1978]. Therefore, there is little Hex B activity in the Sandhoff disease heterozygotes, and even less in the homozygotes.

As noted earlier, changes in apparent Hex A activity can cause problems in the diagnosis of TSD heterozygotes. For example, the same leukocyte sample could yield results for percent Hex A that differ by 10 percentage points when different amounts of sample are used in the assay. Such a difference could lead to misdiagnosis of TSD heterozygosity. Since prenatal diagnosis of TSD is commonly requested by couples who are

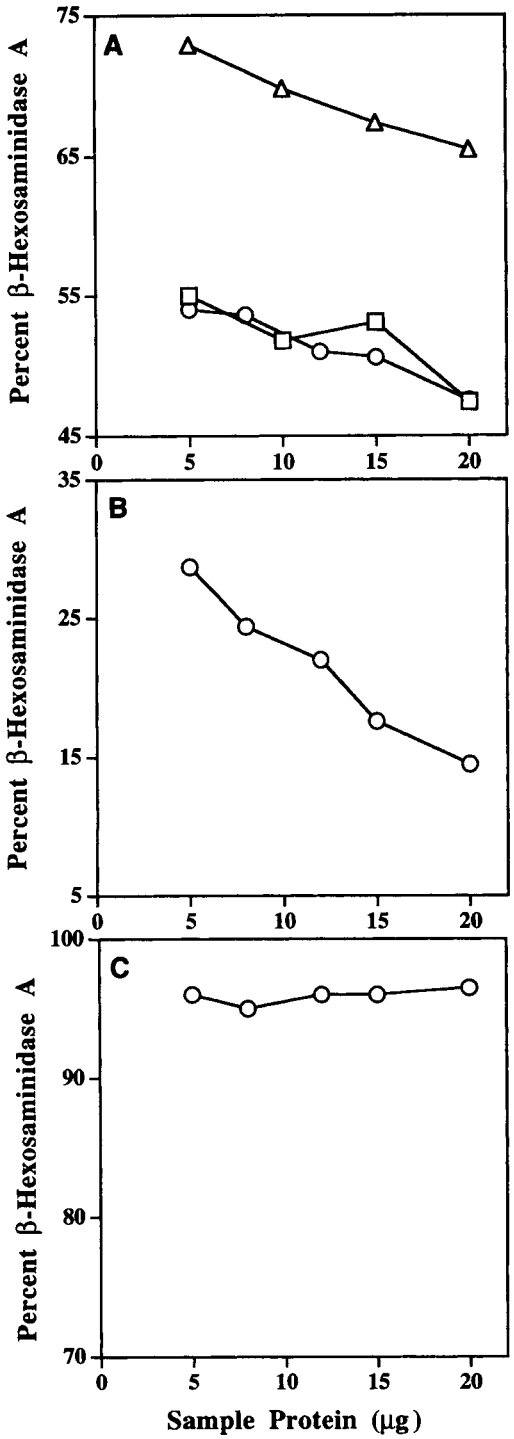

Fig. 1. Effect of sample concentration on apparent Hex A activity in cultured fibroblasts. Fibroblast sonicates were assayed as described in Materials and Methods. Assays were performed at various concentrations of sample protein, as indicated. Shown are results obtained for three different normal controls (**A**), one TSD fibroblast culture (**B**), and one Sandhoff disease fibroblast culture (**C**).

at risk of having a child with TSD (i.e., both members of the couple are TSD heterozygotes or are inconclusive with respect to their carrier status), we were interested in determining if Hex from samples commonly used for TSD prenatal diagnosis also has this unusual thermo-

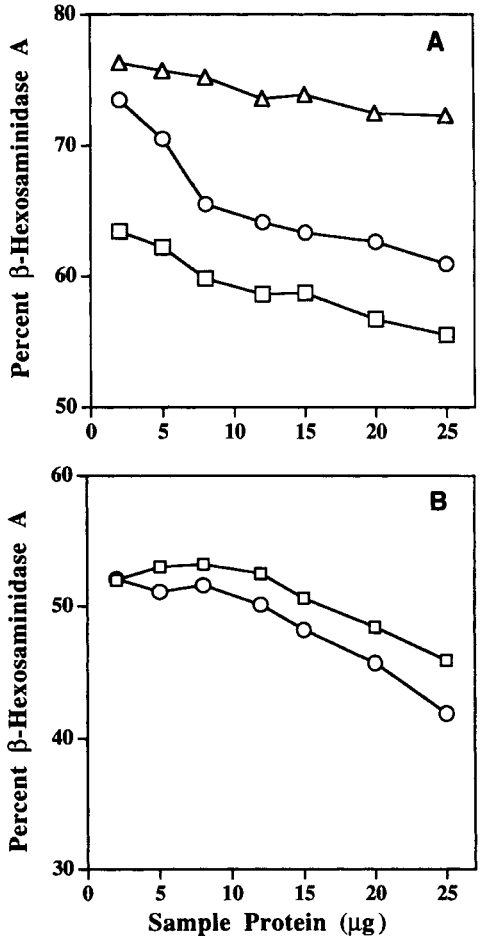

Fig. 2. Effect of sample concentration on apparent Hex A activity in cultured amniotic fluid cells and chorionic villus sample cells. Three different amniotic fluid cell cultures (**A**) and two chorionic villus cell cultures (**B**) were assayed as described for Figure 1.

lability property. As shown in Figure 2A, the apparent percent Hex A from cultured AFCs was also sensitive to sample concentration, although to varying extents in the different cell lines tested. Cultured CVS cells were analyzed in the same way, and here, too, the apparent percent Hex A varied according to the amount of sample used in the assay (Fig. 2B).

In our previous study on leukocyte Hex, we determined that the apparent effects on Hex A activity were really due to changes in Hex B activity [Prence et al., 1993]. Our results on Sandhoff disease fibroblasts indicated that this also may be the case for cultured cells. One additional way to test for effects on Hex A is to perform a direct assay of Hex A using the sulfated substrate, MUGS, which is not significantly hydrolyzed by Hex B [Kresse et al., 1981; Bayleran et al., 1981; Kytzia and Sandhoff, 1984; Ben-Yoseph et al., 1985]. As shown in Figure 3, when the specific assay for Hex A was performed, there was *no* effect of sample concentration on Hex A activity of normal, TSD, or Sandhoff disease fibroblasts.

We speculated earlier that the observed effects on Hex activity might be due to the action of some cellular component that serves to protect Hex B from heat-

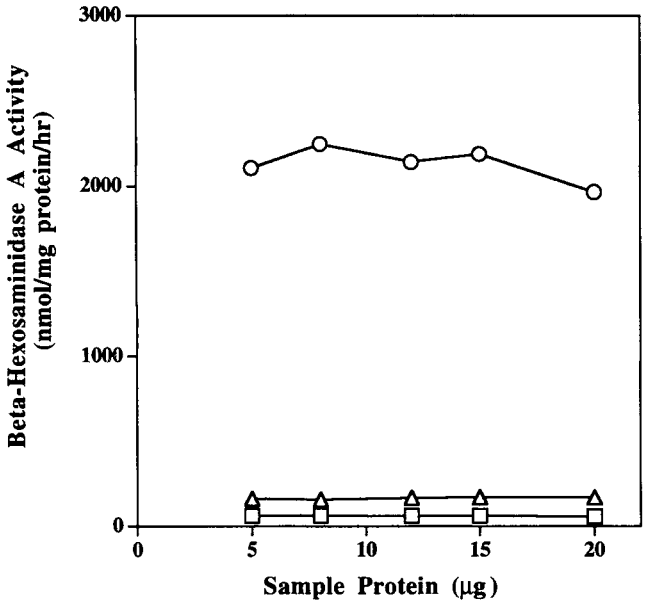

Fig. 3. Direct analysis of Hex A at different sample concentrations. Hex A was assayed directly using the MUGS substrate at various amounts of sample protein, as indicated. Shown are results obtained with normal control (○), TSD fibroblasts (△), and Sandhoff disease fibroblasts (□).

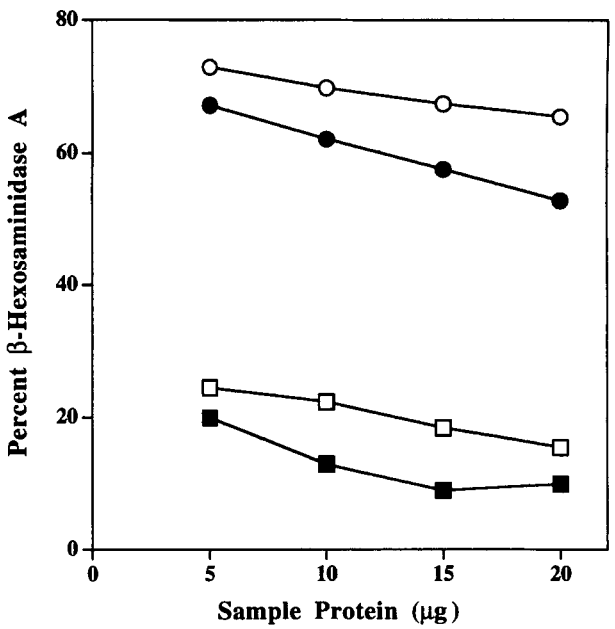

Fig. 4. Assay of beta-hexosaminidase in dialyzed fibroblast sonicates. Normal control and TSD fibroblast sonicates were dialyzed against distilled, deionized water overnight at 4°C. The next day, the heat-inactivation assay was performed as described in Materials and Methods on both the dialyzed samples and the nondialyzed matched samples. Shown are the percent Hex A results obtained for the normal control (circles) and TSD (squares) samples in the nondialyzed (open symbols) and dialyzed (solid symbols) samples.

inactivation [Prence et al., 1993]. That component would be limiting at low sample concentrations, thereby making Hex B less resistant to heat inactivation. In an initial attempt to identify such an effector, we dialyzed fibroblast sonicates prior to performing the Hex heat-inactivation assay. This strategy was used to determine if the proposed effector was a small low molecular weight molecule. However, as shown in Figure 4, dialysis had no significant effect on the change in apparent Hex A activity observed with varying sample concentrations in both normal control (Fig. 4, circles) and TSD (Fig. 4, squares) fibroblast assays.

DISCUSSION

These data document an unusual effect of sample concentration on the apparent activity of Hex A in cultured cells when measured by the commonly-used heat-inactivation assay. With increasing sample concentration, the percent Hex A decreased significantly when measured in normal control and TSD fibroblasts, as well as in cultured AFC and CVS cells. These results on cultured cells are similar to those previously obtained with leukocytes [Prence et al., 1993].

As in our previous study on leukocytes, our data indicate that Hex B, and not Hex A, was affected by the change in sample concentration. The lack of effect on apparent Hex A activity when Sandhoff disease fibroblasts were assayed provided the first evidence that this is the case. Sandhoff disease fibroblasts have little to no Hex B activity; therefore, the lack of change in Hex activity with different sample concentrations can be correlated with a lack of Hex B. Secondly, when Hex A of normal fibroblasts was assayed directly with the sulfated substrate, there was no effect of sample concentration on Hex A activity, independently verifying that the observations made with the heat-inactivation assay were not due to alterations in Hex A activity.

We postulated that the change in leukocyte Hex B activity was due to decreased thermal stability of Hex B at low sample concentrations [Prence et al., 1993]. The reasons for such a change in stability are unclear. It is possible that another cellular component protects Hex B from heat denaturation, and that component may not be present in sufficient quantities to work effectively at low sample concentrations. Our first attempt to identify such an effector was to determine if the observed phenomenon would be affected by dialysis of the sample. If the effector was a small, soluble molecule, it would be removed by dialysis, and large differences between dialyzed and nondialyzed samples would be expected. However, we found no significant difference between the two samples with respect to Hex activity. Other possibilities for a factor that could cause the effects observed in this study include a protein, a large peptide, a lipid, or a combination of two or more components that cannot be removed by dialysis.

The variation in Hex B activity observed with different sample concentrations in AFCs and CVS cells raises the concern of false-positive and false-negative results in clinical testing. Erroneous results are less likely to occur in the context of testing for TSD using cultured cells relative to carrier screening using leukocytes because of the large differences in percent Hex A between affected vs. unaffected individuals compared to the smaller dif-

ference in the percent Hex A between heterozygotes and noncarriers. Nonetheless, the possibility of misdiagnosis is real. Our data argue for rigorous monitoring of sample concentrations for prenatal testing as well as for leukocyte TSD heterozygote screening. Furthermore, an independent methodology such as the MUGS assay, electrophoretic separation of Hex A and Hex B, or, in many instances, mutational analysis, should be used together with the heat-inactivation assay in the evaluation of fetuses or persons with possible TSD. The existence of benign, pseudodeficiency alleles at the Hex alpha subunit locus that behave like disease-causing alleles in the standard Hex assay [Zlogotora and Bach, 1983; Triggs-Raine et al., 1992; Cao et al., 1993; Thomas, 1994] also argues for additional testing, such as mutational analysis, in cultured cells of fetuses or individuals who are at risk for TSD.

REFERENCES

Bayleran J, Hechtman P, Saray W (1981): Synthesis of 4-methylumbelliferyl-beta-D-N-acetylglucosamine-6-sulfate and its use in classification of G_{M2} gangliosidosis phenotypes. Clin Chim Acta 143:73–89.

Ben-Yoseph Y, Reid JE, Shapiro B, Nadler HL (1985): Diagnosis and carrier detection of Tay-Sachs disease: Direct determination of hexosaminidase A using 4-methylumbelliferyl derivatives of beta-N-acetylglucosamine-6-sulfate. Am J Hum Genet 37:733–748.

Cao Z, Natowicz MR, Kaback MM, Lim-Steele JST, Prence E, Chabot T, Triggs-Raine B (1993): A second mutation associated with apparent beta-hexosaminidase A pseudodeficiency: Identification and frequency estimation. Am J Hum Genet 53:1198–1205.

Gravel RA, Clarke JTR, Kaback MM, Mahuran D, Sandhoff D, Suzuki K (1995): The G_{M2} gangliosidoses. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds): "The Metabolic and Molecular Bases of Inherited Disease." New York: McGraw-Hill, pp 2839–2879.

Kaback MM, Shapiro LJ, Hirsch P, Roy C (1977): Tay-Sachs disease heterozygote detection: A quality control study. Prog Clin Biol Res 18:267–279.

Kaback M, Lim-Steele J, Dabholkar D, Brown D, Levy N, Zeiger K (1993): Tay-Sachs disease—Carrier screening, prenatal diagnosis, and the molecular era. JAMA 270:2307–2315.

Kolodny EH (1972): Sandhoff's disease: Studies on the enzyme defect in homozygotes and detection of heterozygotes. In Volk BW (ed): "Sphingolipids, Sphingolipidoses and Allied Disorders." New York: Plenum Publishing Corp., pp 321–341.

Kresse H, Fuchs W, Glossl J, Holtfrerich D, Gilberg W (1981): Liberation of N-acetylglucosamine-6-sulfate by human N-acetylhexosaminidase A. J Biol Chem 256:12926–12932.

Kytzia H-J, Sandhoff K (1984): Evidence for two different active sites on human beta-hexosaminidase. J Biol Chem 260:7568–7572.

Lowden JA, Ives EJ, Keene DL, Burton AL, Skomorowski MA, Howard F (1978): Carrier detection in Sandhoff's disease. Am J Hum Genet 30:38–45.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the Folin reagent. J Biol Chem 193:265–275.

Prence EM, Natowicz MR, Zalewski I (1993): Unusual thermolability properties of leukocyte beta-hexosaminidase: Implications in screening for carriers of Tay-Sachs disease. Clin Chem 39:1811–1814.

Shapira E, Blitzer MG, Miller JB, Africk DK (1989): "Biochemical Genetics: A Laboratory Manual." New York: Oxford University Press, pp 30–35.

Thomas G (1994): Pseudodeficiencies of lysosomal hydrolases. Am J Hum Genet 54:934–940.

Triggs-Raine BL, Mules EH, Kaback MM, Lim-Steele JST, Dowling CE, Akerman BR, Natowicz MR, Grebner EE, Navon R, Welch JP, Greenberg CR, Thomas GH, Gravel RH (1992): A pseudodeficiency allele common in non-Jewish Tay-Sachs carriers: Implications for carrier screening. Am J Hum Genet 51:793–801.

Zlogotora J, Bach G (1983): Deficiency of lysosomal hydrolases in apparently healthy individuals. Am J Med Genet 14:73–80.